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(54) GLUCOSE DEHYDROGENASE

(57) Modified water-soluble glucose dehydrogenases having pyrrolo-quinoline quinone as a coenzyme are provided wherein at least one amino acid residue is replaced by another amino acid residue in a specific region. Modified water-soluble PQQGDHs of the present invention have improved affinity for glucose.

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Description

TECHNICAL FIELD

[0001] The present invention relates to the preparation of glucose dehydrogenases having pyrrolo-quinoline quinone as a coenzyme (PQQGDH) and their use for glucose assays.

BACKGROUND ART

[0002] Blood glucose is an important marker for diabetes. In the fermentative production using microorganisms, glucose levels are assayed for monitoring the process. Conventional glucose assays were based on enzymatic methods using a glucose oxidase (GOD) or glucose-6-phosphate dehydrogenase (G6PDH). However, GOD-based assays required addition of a catalase or peroxidase to the assay system in order to quantitate the hydrogen peroxide generated by glucose oxidation reaction. G6PDHs have been used for spectrophotometric glucose assays, in which case a coenzyme NAD(P) had to be added to the reaction system.

[0003] Accordingly, an object of the present invention is to provide a modified water-soluble PQQGDH with improved affinity for glucose. Another object of the present invention is to provide a modified water-soluble PQQGDH with high selectivity for glucose in order to increase the sensitivity for measuring blood glucose levels.

DISCLOSURE OF THE INVENTION

[0004] We found that PQQGDHs with high affinity for glucose are useful as novel enzymes alternative to the enzymes that have been used for enzymatic glucose assays.

[0005] PQQGDHs are glucose dehydrogenases having pyrrolo-quinoline quinone as a coenzyme, which catalyze the reaction in which glucose is oxidized to produce gluconolactone.

[0006] PQQGDHs are known to include membrane-bound enzymes and water-soluble enzymes. Membrane-bound PQQGDHs are single peptide proteins having a molecular weight of about 87 kDa and widely found in various gram-negative bacteria. For example, see AM. Cleton-Jansen et al., J. Bacteriol. (1990) 172, 6308-6315. On the other hand, water-soluble PQQGDHs have been identified in several strains of *Acinetobacter calcoaceticus* (Biosci. Biotech. Biochem. (1995), 59(8), 1548-1555), and their structural genes were cloned to show the amino acid sequences (Mol. Gen. Genet. (1989), 217:430-436). The water-soluble PQQGDH derived from *A. calcoaceticus* is a homodimer having a molecular weight of about 50 kDa. It has little homology in primary structure of protein with other PQQ enzymes.

[0007] Recently, the results of an X-ray crystal structure analysis of this enzyme were reported to show the higher-order structure of the enzyme including the active center (J. Mol. Biol., 289, 319-333 (1999), The crystal structure of the apo form of the soluble quinoprotein glucose dehydrogenase from Acinetobacter calcoaceticus reveals a novel internal conserved sequence repeat; A. Oubrie et al., The EMBO Journal, 18(19) 5187-5194 (1999), Structure and mechanism of soluble quinoprotein glucose dehydrogenase, A. Oubrie et al., PNAS, 96(21), 11787-11791 (1999), Active-site structure of the soluble quinoprotein glucose dehydrogenase complexed with methylhydrazine; A covalent cofactor-inhibitor complex, A. Oubrie et al.). These papers showed that the water-soluble PQQGDH is a β-propeller protein composed of six W-motifs.

[0008] As a result of careful studies to develop a modified PQQGDH that can be applied to clinical tests or food analyses by improving the conventional water-soluble PQQGDH to increase the affinity for glucose, we succeeded in obtaining an enzyme with high affinity for glucose by introducing an amino acid change into a specific region of the water-soluble PQQGDH.

[0009] Accordingly, the present invention provides a modified water-soluble glucose dehydrogenase having pyrrolo-quinoline quinone as a coenzyme characterized in that at least one amino acid residue in a natural water-soluble glucose dehydrogenase is replaced by another amino acid residue and it has improved affinity for glucose as compared with the natural water-soluble glucose dehydrogenase. The modified PQQGDH of the present invention has a Km value for glucose lower than the Km value of the natural PQQGDH, preferably less than 20 mM, more preferably less than 10 mM.

[0010] Preferably, the modified glucose dehydrogenase of the present invention has increased affinity for glucose though its affinities for other sugars are unchanged or decreased, whereby it has higher selectivity for glucose than the natural water-soluble glucose dehydrogenase. Especially, the reactivity against lactose or maltose is decreased from that of the wild-type in contrast to the reactivity to glucose. When the reactivity against glucose is supposed to be 100%, the activity to lactose or maltose is preferably 60% or less, more preferably 50% or less, still more preferably 40% or less.

[0011] In an embodiment of the PQQ glucose dehydrogenase of the present invention, at least one amino acid residue in the region corresponding to residues 268-289 or 448-468 in the water-soluble PQQGDH derived from *Aci-*

netobacter calcoaceticus is replaced by another amino acid residue, i.e. an amino acid residue other than the relevant amino acid residue in the natural PQQ glucose dehydrogenase. The amino acid numbering herein starts from the initiator methionine as the +1 position.

[0012] The term "correspond to" used herein with reference to amino acid residues or regions means that some amino acid residues or regions have an equivalent function in two or more structurally similar but distinct proteins. For example, any region in water-soluble PQQGDHs derived from other organisms than *Acinetobacter calcoaceticus* is said to "correspond to the region defined by residues 268-289 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus*" if this region has a high similarity in the amino acid sequence to the region defined by residues 268-289 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* and this region is reasonably considered from the secondary structure of the protein to have the same function in that protein. In addition, the 10th amino acid residue in this region is said to "correspond to the 277th residue in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus*".

[0013] In preferred modified PQQGDHs of the present invention, at least one amino acid residue corresponding to glutamate 277, isoleucine 278, asparagine 462, asparagine 452, lysine 455, aspartate 456, aspartate 457 or aspartate 448 in the amino acid sequence shown as SEQ ID NO: 1 is replaced by another amino acid residue.

[0014] In more preferred modified PQQGDHs of the present invention, glutamate 277 is replaced by an amino acid residue selected from the group consisting of alanine, asparagine, lysine, aspartate, histidine, glutamine, valine and glycine, or isoleucine 278 is replaced by phenylalanine in the amino acid sequence shown as SEQ ID NO: 1.

[0015] In another aspect, modified PQQGDHs of the present invention comprise the sequence:

Xaa8 Thr Ala Gly Xaa1 Val Gln Xaa2 Xaa3 Xaa4 Gly Ser Val Thr Xaa5 Thr Leu Glu Asn Pro Gly

wherein Xaa1, Xaa2, Xaa3, Xaa4, Xaa5 and Xaa8 represent any natural amino acid residue, provided that when Xaa1 represents Asn, Xaa2 represents Lys, Xaa3 represents Asp, Xaa4 represents Asp and Xaa5 represents Asn, then Xaa8 does not represent Asp.

[0016] In another aspect, modified PQQGDHs of the present invention comprise the sequence:

Ser Glu Gln Gly Pro Asn Ser Asp Asp Xaa6 Xaa7 Asn Leu Ile Val Lys Gly Gly Asn Tyr Gly Trp

wherein Xaa6 and Xaa7 represent any natural amino acid residue, provided that when Xaa6 represents Glu, Xaa7 does not represent Ile.

[0017] The present invention also provides a gene encoding any of the modified glucose dehydrogenases described above, a vector containing said gene and a transformant containing said gene, as well as a glucose assay kit and a glucose sensor comprising a modified glucose dehydrogenase of the present invention.

[0018] Enzyme proteins of modified PQQGDHs of the present invention have high affinity for glucose and high oxidation activity for glucose so that they can be applied to highly sensitive and highly selective glucose assays.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019]

FIG. 1 shows the structure of the plasmid pGB2 used in the present invention.
FIG. 2 shows a scheme for preparing a mutant gene encoding a modified enzyme of the present invention.
FIG. 3 shows a glucose assay using a modified PQQGDH of the present invention.

THE MOST PREFERRED EMBODIMENTS OF THE INVENTION

Structure of modified POOGDHs

[0020] We introduced random mutations into the coding region of the gene encoding the water-soluble PQQGDH by error-prone PCR to construct a library of water-soluble PQQGDHs carrying amino acid changes. These genes were transformed into *E. coli* and screened for the activity of the PQQGDHs against glucose to give a number of clones that express PQQGDHs having comparable activities for 20 mM glucose and 100 mM glucose and improved reactivity against low-level glucose as compared with that of the wild-type enzyme.

[0021] Analysis of the nucleotide sequence of one of these clones showed that Glu 277 had been changed to Gly. When this amino acid residue was replaced by various other amino acid residues, excellent mutant enzymes with improved affinity for glucose as compared with that of the wild type water-soluble PQQGDH were obtained in every case.

[0022] Then, site-specific mutations were introduced into other residues near the 277th residue and the affinity for

glucose was determined. Modified enzymes carrying Ile278Phe and Asn279His in the region defiend by residues 268-289 were prepared and assayed for the activity to show that these modified enzymes had high affinity for glucose.

[0023] A number of clones obtained as above were further screened for clones that express PQQGDHs having activity for 20 mM glucose comparable to that of the wild-type PQQGDH but activity for 20 mM lactose lower than that of the wild-type PQQGDH.

[0024] Analysis of the nucleotide sequence of one of these clones showed that Asn 452 had been changed to Asp. When this residue was replaced by threonine, lysine, isoleucine, histidine or aspartate, excellent mutant enzymes with improved selectivity for glucose as compared with that of the wild type water-soluble PQQGDH were obtained in every case. Mutations were also introduced into other residues near the 452nd residue in the same manner. Mutant enzymes carrying Lys455Ile, Asp456Asn, Asp457Asn, Asn462Asp, Asp448Asn were constructed. As a result, all the mutant enzymes were found to have improved selectivity for glucose as shown in Table 4.

[0025] In preferred PQQ glucose dehydrogenases of the present invention, at least one amino acid residue is replaced by another amino acid residue in the region corresponding to residues 448-468 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus*. In preferred modified PQQGDHs of the present invention, at least one amino acid residue corresponding to asparagine 462, lysine 452, aspartate 456, aspartate 457 or aspartate 448 in the amino acid sequence shown as SEQ ID NO: 1 is replaced by another amino acid residue.

[0026] In another aspect, modified PQQGDHs of the present invention comprise the sequence:

Xaa8 Thr Ala Gly Xaa1 Val Gln Xaa2 Xaa3 Xaa4 Gly Ser Val Thr Xaa5 Thr Leu Glu Asn Pro Gly

wherein Xaa1, Xaa2, Xaa3, Xaa4, Xaa5 and Xaa8 represent any natural amino acid residue, provided that when Xaa1 represents Asn, Xaa2 represents Lys, Xaa3 represents Asp, Xaa4 represents Asp and Xaa5 represents Asn, then Xaa8 does not represent Asp.

[0027] In other preferred PQQ glucose dehydrogenases of the present invention, at least one amino acid residue is replaced by another amino acid residue in the region corresponding to residues 268-289 in the amino acid sequence shown as SEQ ID NO: 1. In especially preferred modified PQQGDHs of the present invention, glutamate 277 is replaced by an amino acid residue selected from the group consisting of alanine, asparagine, lysine, aspartate, histidine, glutamine, valine and glycine, or isoleucine 278 is replaced by phenylalanine in the amino acid sequence shown as SEQ ID NO: 1.

[0028] In another aspect, modified PQQGDHs of the present invention comprise the sequence:

Ser Glu Gln Gly Pro Asn Ser Asp Asp Xaa6 Xaa7 Asn Leu Ile Val Lys Gly Gly Asn Tyr Gly Trp

wherein Xaa6 and Xaa7 represent any natural amino acid residue, provided that when Xaa6 represents Glu, Xaa7 does not represent Ile.

[0029] In modified glucose dehydrogenases of the present invention, other amino acid residues may be partially deleted or substituted or other amino acid residues may be added so far as glucose dehydrogenase activity is retained.

[0030] Those skilled in the art can also replace an amino acid residue in water-soluble PQQGDHs derived from other bacteria according to the teaching herein to obtain modified glucose dehydrogenases with improved affinity for glucose. Particularly, amino acid residues corresponding to glutamate 277, isoleucine 278, asparagine 462, lysine 452, aspartate 455, aspartate 456, aspartate 457 and aspartate 448 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* can be readily identified by comparing the primary structures of proteins in alignment or comparing the secondary structures predicted from the primary structures of the enzymes. Modified glucose dehydrogenases with improved affinity for substrate can be obtained by replacing such amino acid residues according to the present invention. These modified glucose dehydrogenases are also within the scope of the present invention.

Process for preparing modified POOGDHs

[0031] The sequence of the gene encoding the wild-type water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* is defined by SEQ ID NO: 2.

[0032] Genes encoding modified PQQGDHs of the present invention can be constructed by replacing the nucleotide sequence encoding a specific amino acid residue in the gene encoding the wild-type water-soluble PQQGDH by the nucleotide sequence encoding an amino acid residue to be substituted. Various techniques for such site-specific nucleotide sequence substitution are known in the art as described in Sambrook et al., "Molecular Cloning: A Laboratory Manual", Second Edition, 1989, Cold Spring Harbor Laboratory Press, New York, for example.

[0033] Thus obtained mutant gene is inserted into a gene expression vector (for example, a plasmid) and transformed into an appropriate host (for example, *E. coli*). A number of vector/host systems for expressing a foreign protein are known and various hosts such as bacteria, yeasts or cultured cells are suitable.

[0034] Random mutations are introduced by error-prone PCR into a target region to construct a gene library of modified water-soluble PQQGDHs carrying mutations in the target region. These genes are transformed into *E. coli* to screen each clone for the affinity of the PQQGDH for glucose. Water-soluble PQQGDHs are secreted into the periplasmic space when they are expressed in *E. coli*, so that they can be easily assayed for enzyme activity using the *E. coli* cells. This library is combined with a PMS-DCIP dye in the presence of 20 mM glucose to visually determine the PQQGDH activity so that clones showing activity comparable to the activity for 100 mM glucose are selected and analyzed for the nucleotide sequence to confirm the mutation.

[0035] In order to obtain modified PQQGDHs with improved selectivity for glucose, this library is combined with a PMS-DCIP dye to visually determine the PQQGDH activity so that clones showing activity for 20 mM glucose comparable to that of the wild-type PQQGDH but activity for 20 mM lactose lower than that of the wild-type PQQGDH are selected and analyzed for the nucleotide sequence to confirm the mutation.

[0036] Thus obtained transformed cells expressing modified PQQGDHs are cultured and harvested by centrifugation or other means from the culture medium, and then disrupted with a French press or osmotically shocked to release the periplasmic enzyme into the medium. The enzyme may be ultracentrifuged to give a water-soluble PQQGDH-containing fraction. Alternatively, the expressed PQQGDH may be secreted into the medium by using an appropriate host/vector system. The resulting water-soluble fraction is purified by ion exchange chromatography, affinity chromatography, HPLC and the like to prepare a modified PQQGDH of the present invention.

Method for assaying enzyme activity

[0037] PQQGDHs of the present invention associate with PQQ as a coenzyme in catalyzing the reaction in which glucose is oxidized to produce gluconolactone.

[0038] The enzyme activity can be assayed by using

the color-developing reaction of a redox dye to measure the amount of PQQ reduced with PQQGDH-catalyzed oxidation of glucose. Suitable color-developing reagents include PMS (phenazine methosulfate)-DCIP (2,6-dichlorophenolindophenol), potassium ferricyanide and ferrocene, for example.

Affinity for glucose

[0039] Modified PQQGDHs of the present invention have greatly improved affinity for glucose as compared with that of the wild type. Thus, modified PQQGDHs have a Km value for glucose that is greatly lower than the Km value for glucose of the wild-type PQQGDH. Among modified PQQGDHs, the Glu277Lys variant has a Km value for glucose of 8.8 mM and a maximum activity comparable to that of the wild-type enzyme so that it has improved reactivity against glucose at low levels.

[0040] Therefore, assay kits or enzyme sensors prepared with modified enzymes of the present invention have the excellent advantages that they can detect glucose at low levels because of the high sensitivity for glucose assays.

Evaluation method of selectivity

[0041] Selectivity for glucose of PQQGDHs of the present invention can be evaluated by assaying the enzyme activity as described above using various sugars such as 2-deoxy-D-glucose, mannose, allose, 3-o-methyl-D-glucose, galactose, xylose, lactose and maltose as substrates and determining the relative activity to the activity for glucose.

Glucose assay kit

[0042] The present invention also relates to a glucose assay kit comprising a modified PQQGDH according to the present invention. The glucose assay kit of the present invention comprises a modified PQQGDH according to the present invention in an amount enough for at least one run of assay. In addition to the modified PQQGDH according to the present invention, the kit typically comprises a necessary buffer for the assay, a mediator, standard glucose solutions for preparing a calibration curve and instructions. Modified PQQGDHs according to the present invention can be provided in various forms such as freeze-dried reagents or solutions in appropriate preservative solutions. Modified PQQGDHs according to the present invention are preferably provided in the form of a holoenzyme, though they may also be provided as an apoenzyme and converted into a holoenzyme before use.

Glucose sensor

[0043] The present invention also relates to a glucose sensor using a modified PQQGDH according to the present

invention. Suitable electrodes include carbon, gold, platinum and the like electrodes, on which an enzyme of the present invention is immobilized by using a crosslinking agent; encapsulation in a polymer matrix; coating with a dialysis membrane; using a photo-crosslinkable polymer, an electrically conductive polymer or a redox polymer; fixing the enzyme in a polymer or adsorbing it onto the electrode with an electron mediator including ferrocene or its derivatives; or any combination thereof. Modified PQQGDHs of the present invention are preferably immobilized in the form of a holoenzyme on an electrode, though they may be immobilized as an apoenzyme and PQQ may be provided as a separate layer or in a solution. Typically, modified PQQGDHs of the present invention are immobilized on a carbon electrode with glutaraldehyde and then treated with an amine-containing reagent to block glutaraldehyde.

[0044] Glucose levels can be measured as follows. PQQ, $CaCl_2$ and a mediator are added to a thermostat cell containing a buffer and kept at a constant temperature. Suitable mediators include, for example, potassium ferricyanide and phenazine methosulfate. An electrode on which a modified PQQGDH of the present invention has been immobilized is used as a working electrode in combination with a counter electrode (e.g. a platinum electrode) and a reference electrode (e.g. an Ag/AgCl electrode). After a constant voltage is applied to the carbon electrode to reach a steady current, a glucose-containing sample is added to measure the increase in current. The glucose level in the sample can be calculated from a calibration curve prepared with glucose solutions at standard concentrations.

[0045] The disclosures of all the patents and documents cited herein are entirely incorporated herein as reference. The present application claims priority based on Japanese Patent Applications Nos. 1999-124285 and 2000-9137, the disclosure of which is entirely incorporated herein as reference.

[0046] The following examples further illustrate the present invention without, however, limiting the same thereto.

Example 1

Construction and screening of a mutant PQQGDH gene library:

[0047] The plasmid pGB2 was obtained by inserting the structural gene encoding the PQQGDH derived from *Acinetobacter calcoaceticus* into the multicloning site of the vector pTrc99A (Pharmacia) (Fig. 1). This plasmid was used as a template to introduce random mutations into various regions by error-prone PCR. The PCR reaction was carried out in a solution having the composition shown in Table 1 under the conditions of 94°C for 3 minutes, 30 cycles of 94°C for 3 minutes, 50°C for 2 minutes and 72°C for 2 minutes, and finally 72°C for 10 minutes.

Table 1

TaqDNA polymerase (5U/μl)	0.5 μl
Template DNA	1.0 μl
Forward primer ABF	4.0 μl
Reverse primer ABR	4.0 μl
10 x Taq polymerase buffer	10.0 μl
1M β-mercaptoethanol	1.0 μl
DMSO	10.0 μl
5 mM $MnCl_2$	10.0 μl
10 mM dGTP	2.0 μl
2 mM dATP	2.0 μl
10 mM dCTP	2.0 μl
10 mM dTTP	2.0 μl
H_2O	51.5 μl
	100.0 μl

[0048] The resulting mutant water-soluble PQQGDH library was transformed into *E. coli* and each colony formed was transferred to a microtiter plate. The colony was further replica-plated on a first plate containing 10 mM glucose and PMS-DCIP and a second plate containing 100 mM glucose and PMS-CDIP, and both were visually evaluated for the PQQGDH activity. A number of clones showing comparable PQQGDH activities in both plates were obtained.

[0049] One of these clones was randomly selected and analyzed for the nucleotide sequence to show that glutamate 277 had been changed to glycine.

Example 2

[0050] Each colony obtained in Example 1 was transferred to a microtiter plate. The colony was replica-plated on a first plate containing 20 mM glucose and PMS-DCIP and a second plate containing 20 mM lactose and PMS-CDIP, and both were visually evaluated for the PQQGDH activity. A number of clones showing a greatly lower activity for lactose than glucose in both plates were obtained.

[0051] One of these clones was randomly selected and analyzed for the nucleotide sequence to show that asparagine 452 had been changed to aspartate.

Example 3

Construction of modified PQQGDH genes:

[0052] Based on the structural gene of the PQQGDH derived from *Acinetobacter calcoaceticus* shown as SEQ ID NO: 2, the nucleotide sequence encoding glutamate 277 or isoleucine 278 was replaced by the nucleotide sequences encoding given amino acid residues by site-directed mutagenesis according to a standard method as shown in Fig. 2 using the plasmid pGB2. Table 2 shows the sequences of the synthetic oligonucleotide target primers used for mutagenesis. In Table 2, "E277A" means that glutamate 277 is replaced by aspartate, for example.

Table 2

E277A	5'- GAG GTT AAT TGC ATC GTC AGA G -3'
E277N	5'- C AAT GAG GTT AAT GTT ATC GTC AGA GTT TG-3'
E277K	5'- GAG GTT AAT ATC ATC GTC AGA G -3'
E277D	5'- GAG GTT AAT TTT ATC GTC AGA G -3'
E277H	5'- C AAT GAG GTT AAT GTG ATC GTC AGA GTT TG-3'
E277Q	5'- GAG GTT AAT TTG ATC GTC AGA G -3'
E277V	5'- C AAT GAG GTT AAT TAC ATC GTC AGA GTT TG-3'
E277G	5'- GAG GTT AAT TCC ATC GTC AGA G -3'
I278F	5'- C AAT GAG GTT GAA TTC ATC GTC AGA G -3'
N279H	5'-GAC AAT GAG GTC AAT TTC ATC GTC AGA GTT -3'

[0053] A KpnI-HindIII fragment containing a part of the gene encoding the PQQGDH derived from *Acinetobacter calcoaceticus* was integrated into the vector plasmid pKF18k (Takara Shuzo Co., Ltd.) and used as a template. Fifty fmols of this template, 5 pmol of the selection primer attached to the Mutan™-Express Km Kit (Takara Shuzo Co., Ltd.) and 50 pmol of the phosphorylated target primer were mixed with the annealing buffer attached to the kit in an amount equivalent to 1/10 of the total volume (20 μl), and the mixture was heated at 100°C for 3 minutes to denature the plasmid into a single strand. The selection primer serves for reversion of dual amber mutations on the kanamycin-resistance gene of pKF18k. The mixture was placed on ice for 5 minutes to anneal the primers. To this mixture were added 3 μl of the extension buffer attached to the kit, 1 μl of T4 DNA ligase, 1 μl of T4 DNA polymerase and 5 μl of sterilized water to synthesize a complementary strand.

[0054] The synthetic strand was transformed into a DNA mismatch repair-deficient strain *E. coli* BMH71-18mutS and shake-cultured overnight to amplify the plasmid.

[0055] Then, the plasmid copies were extracted from the cultures and transformed into *E. coil* MV1184 and then extracted from the colonies. These plasmids were sequenced to confirm the introduction of the intended mutations. These fragments were substituted for the KpnI-HindIII fragment of the gene encoding the wild-type PQQGDH on the plasmid pGB2A to construct genes for modified PQQGDHs.

[0056] An oligonucleotide target primer of the sequence: 5'-C ATC TTT TTG GAC ATG TCC GGC AGT AT-3' was synthesized in the same manner to substitute histidine for asparagine 452. Site-directed mutagenesis was performed by the method shown in Fig. 2 using the plasmid pGB2. Genes for modified PQQGDHs carrying mutations Asp448Asn, Asn452Asp, Asn452His, Asn452Lys, Asn452Thr, Asn452Ile, Lys455Ile, Asp456Asn, Asp457Asn and Asn462Asp were also constructed.

Example 4

Preparation of modified enzymes

[0057] The gene encoding the wild-type or each modified PQQGDH was inserted into the multicloning site of an *E. coil* expression vector pTrc99A (Pharmacia), and the resulting plasmid was transformed into the *E. coli* strain DH5α. The transformant was shake-cultured at 37°C overnight on 450 ml of L medium (containing 50 μg/ml of ampicillin) in a Sakaguchi flask, and inoculated on 7 l of L medium containing 1 mM $CaCl_2$ and 500 μM PQQ. About 3 hours after starting cultivation, isopropyl thiogalactoside was added at a final concentration of 0.3 mM, and cultivation was further continued for 1.5 hours. The cultured cells were harvested from the medium by centrifugation (5,000 x g, 10 min, 4°C), and washed twice with a 0.85% NaCl solution. The collected cells were disrupted with a French press, and centrifuged (10,000 x g, 15 min, 4°C) to remove undisrupted cells. The supernatant was ultracentrifuged (160,500 x g (40,000 r. p.m.), 90 min, 4°C) to give a water-soluble fraction, which was used in the subsequent examples as a crude enzyme sample.

[0058] Thus obtained water-soluble fraction was further dialyzed against 10 mM phosphate buffer, pH 7.0 overnight. The dialyzed sample was adsorbed to a cation chromatographic column TSKgel CM-TOYOPEARL 650M (Tosoh Corp.), which had been equilibrated with 10 mM phosphate buffer, pH 7.0. This column was washed with 750 ml of 10 mM phosphate buffer, pH 7.0 and then the enzyme was eluted with 10 mM phosphate buffer, pH 7.0 containing 0-0.2 M NaCl at a flow rate of 5 ml/min. Fractions having GDH activity were collected and dialyzed against 10 mM MOPS-NAOH buffer, pH 7.0 overnight. Thus, an electrophoretically homogeneous modified PQQGDH protein was obtained. This was used in the subsequent examples as a purified enzyme sample.

Example 5

Assay of enzyme activity:

[0059] Enzyme activity was assayed by using PMS (phenazine methosulfate)-DCIP (2,6-dichlorophenolindophenol) in 10 mM MOPS-NaOH buffer (pH 7.0) to monitor changes in the absorbance of DCIP at 600 nm with a spectrophotometer and expressing the reaction rate of the enzyme as the rate of decrease in the absorbance. The enzyme activity for reducing 1 μmol of DCIP in 1 minute was 1 U. The molar extinction coefficient of DCIP at pH 7.0 was 16.3 mM^{-1}.

Example 6

Evaluation of affinity of crude enzyme samples for glucose:

[0060] Each of the crude enzyme samples of the wild-type and modified PQQGDHs obtained in Example 4 was converted into a holoenzyme in the presence of 1 μM PQQ and 1 mM $CaCl_2$ for 1 hour or longer. A 187 μl-aliquot was combined with 3 μl of an activating reagent (prepared from 48 μl of 6 mM DCIP, 8 μl of 600 mM PMS and 16 μl of 10 mM phosphate buffer, pH 7.0) and 10 μl of D-glucose solutions at various concentrations, and assayed for the enzyme activity at room temperature by the method shown in Example 5. The Km was determined by plotting the substrate concentration vs. enzyme activity. The results are shown in Table 3.

Table 3

	Km (mM)
Wild type	26.0
G277A	1.5
G277N	1.2
G277K	8.9
G277D	7.4
G277H	7.7

Table 3 (continued)

	Km (mM)
G277Q	4.3
G277V	2.5
G277G	0.3
I278F	7.0
N279H	15.7
N452T	12.5
N462D	12.2
N462K	11.0
N462Y	20.4

[0061] The Km value of the wild-type PQQGDH for glucose reported to date was about 25 mM. In contrast, all the enzymes constructed here to carry mutations in glutamate 277 and Ile278Phe had a Km value for glucose of less than 10 mM. These results show that modified PQQGDHs of the present invention have high affinity for glucose.

Example 7

Evaluation of affinity of purified enzyme samples for glucose:

[0062] Each of the purified samples of the wild-type enzyme and the modified enzyme Glu277Lys obtained in Example 4 was converted into a holoenzyme in the presence of 1 μM PQQ and 1 mM $CaCl_2$ for 1 hour or longer in the same manner as in Example 6. A 187 μl-aliquot was combined with 3 μl of an activating reagent (prepared from 48 μl of 6 mM DCIP, 8 μl of 600 mM PMS and 16 μl of 10 mM phosphate buffer, pH 7.0) and 10 μl of D-glucose solutions at various concentrations, and assayed for the enzyme activity at room temperature by the method shown in Example 5. The Km and Vmax were determined by plotting the substrate concentration vs. enzyme activity. The Glu277Lys variant had a Km value for glucose of about 8.8 mM and a Vmax value of 3668 U/mg. The Km value of the wild-type PQQGDH for glucose reported to date was about 25 mM with the Vmax value being 2500-7000 U/mg depending on the measurement conditions. These results show that the modified PQQGDH Glu277Lys is an enzyme having remarkably improved affinity for glucose and high activity comparable to that of the wild-type PQQGDH.

Example 8

Evaluation of substrate specificity:

[0063] Crude samples of various modified enzymes were tested for substrate specificity. Each of the crude samples of the wild-type and various modified PQQGDHs was converted into a holoenzyme in the presence of 1 μM PQQ and 1 mM $CaCl_2$ for 1 hour or longer. A 187 μl-aliquot was combined with 3 μl of an activating reagent (containing 6 mM DCIP, 600 mM PMS and 10 mM phosphate buffer, pH 7.0) and a substrate. The substrates tested were 400 mM glucose, lactose and maltose at a final concentration of 20 mM, and each sample was incubated with 10 μl of each substrate at room temperature for 30 minutes and assayed for the enzyme activity in the same manner as in Example 5 to determine the relative activity expressed as the percentage of the activity for glucose. As shown in Table 4, all the modified enzymes of the present invention showed higher selectivity for glucose than that of the wild-type enzyme.

Table 4

	Glucose	Lactose	Maltose
Wild-type	100%	61%	61%
Asp448Asn	100%	48%	36%
Asn452Asp	100%	56%	50%
Asn452His	100%	39%	39%
Asn452Lys	100%	55%	42%
Asn452Thr	100%	42%	30%
Asn452Ile	100%	36%	28%
Lys455Ile	100%	49%	37%

Table 4 (continued)

	Glucose	Lactose	Maltose
Asp456Asn	100%	59%	41%
Asp457Asn	100%	43%	32%
Asn462Asp	100%	52%	41%

Example 9

Glucose assay:

[0064] Modified PQQGDHs were used for assaying glucose. Each of the modified enzymes Glu277Lys and Asn452Thr was converted into a holoenzyme in the presence of 1 μM PQQ and 1 mM $CaCl_2$ for 1 hour or longer, and assayed for the enzyme activity in the presence of glucose at various concentrations as well as 5 μM PQQ and 10 mM $CaCl_2$ by the method described in Example 5 based on changes of the absorbance of DCIP at 600 nm. As shown in Fig. 3, the modified PQQGDH Asn452Thr could be used for assaying glucose in the range of 0.1-20 mM. Similar results were obtained with the modified PQQGDH Glu277Lys.

Example 10

Preparation and evaluation of an enzyme sensor:

[0065] Five units each of the modified enzymes Glu277Lys and Asn452Thr were freeze-dried with 20 mg of carbon paste. After thorough mixing, the mixture was applied only on the surface of a carbon paste electrode preliminarily filled with about 40 mg of carbon paste and polished on a filter paper. This electrode was treated in 10 mM MOPS buffer (pH 7.0) containing 1% glutaraldehyde at room temperature for 30 minutes followed by 10 mM MOPS buffer (pH 7.0) containing 20 mM lysine at room temperature for 20 minutes to block glutaraldehyde. The electrode was equilibrated in 10 mM MOPS buffer (pH 7.0) at room temperature for 1 hour or longer and then stored at 4°C.

[0066] Thus prepared enzyme sensor was used to measure glucose levels. The enzyme sensor having a modified PQQGDH of the present invention immobilized thereon can be used for assaying glucose in the range of 0.1 mM - 5 mM.

INDUSTRIAL APPLICABILITY

[0067] Modified PQQGDHs of the present invention have high affinity for glucose so that they are expected to provide the advantages that assay kits or enzyme sensors prepared with such enzymes can measure glucose at lower levels with remarkably improved sensitivity as compared with conventional natural PQQGDHs.

Sequence Listing

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Lys Ile Leu Arg Val Asn Pro Glu Ser Gly Ser Val Lys Thr Val Phe
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Pro Lys Asp Asn Pro Ser Phe Asn Gly Val Val Ser His Ile Tyr Thr

210 215 220

Leu Gly His Arg Asn Pro Gln Gly Leu Ala Phe Thr Pro Asn Gly Lys

225 230 235 240

Leu Leu Gln Ser Glu Gln Gly Pro Asn Ser Asp Asp Glu Ile Asn Leu

245 250 255

Ile Val Lys Gly Gly Asn Tyr Gly Trp Pro Asn Val Ala Gly Tyr Lys

260 265 270

Asp Asp Ser Gly Tyr Ala Tyr Ala Asn Tyr Ser Ala Ala Ala Asn Lys

275 280 285

Ser Ile Lys Asp Leu Ala Gln Asn Gly Val Lys Val Ala Ala Gly Val

290 295 300

Pro Val Thr Lys Glu Ser Glu Trp Thr Gly Lys Asn Phe Val Pro Pro

305 310 315 320

Leu Lys Thr Leu Tyr Thr Val Gln Asp Thr Tyr Asn Tyr Asn Asp Pro

325 330 335

Thr Cys Gly Glu Met Thr Tyr Ile Cys Trp Pro Thr Val Ala Pro Ser

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Ser Ala Tyr Val Tyr Lys Gly Gly Lys Lys Ala Ile Thr Gly Trp Glu

355 360 365

Asn Thr Leu Leu Val Pro Ser Leu Lys Arg Gly Val Ile Phe Arg Ile

370 375 380

Lys Leu Asp Pro Thr Tyr Ser Thr Thr Tyr Asp Asp Ala Val Pro Met

385 390 395 400

Phe Lys Ser Asn Asn Arg Tyr Arg Asp Val Ile Ala Ser Pro Asp Gly

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Claims

1. A modified water-soluble glucose dehydrogenase having pyrrolo-quinoline quinone as a coenzyme **characterized in that** at least one amino acid residue in a natural water-soluble glucose dehydrogenase is replaced by another amino acid residue and it has improved affinity for glucose as compared with the natural water-soluble glucose dehydrogenase.

2. The modified glucose dehydrogenase of Claim 1 having high selectivity for glucose as compared with the wild-type PQQGDH.

3. A modified glucose dehydrogenase having pyrrolo-quinoline quinone as a coenzyme wherein asparagine 462 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* or an amino acid residue corresponding to said residue is replaced by another amino acid residue.

4. A modified glucose dehydrogenase having pyrroloquinoline quinone as a coenzyme wherein asparagine 452 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* or an amino acid residue corresponding to said residue is replaced by another amino acid residue.

5. A modified glucose dehydrogenase having pyrrolo-quinoline quinone as a coenzyme wherein lysine 455 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* or an amino acid residue corresponding to said residue is replaced by another amino acid residue.

6. A modified glucose dehydrogenase having pyrrolo-quinoline quinone as a coenzyme wherein aspartate 456 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* or an amino acid residue corresponding to said residue is replaced by another amino acid residue.

7. A modified glucose dehydrogenase having pyrroloquinoline quinone as a coenzyme wherein aspartate 457 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* or an amino acid residue corresponding to said residue is replaced by another amino acid residue.

8. A modified glucose dehydrogenase having pyrrolo-quinoline quinone as a coenzyme wherein aspartate 448 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* or an amino acid residue corresponding to said residue is replaced by another amino acid residue.

9. A modified glucose dehydrogenase having pyrrolo-quinoline quinone as a coenzyme wherein at least one amino acid residue is replaced by another amino acid residue in the region corresponding to residues 268-289 or 448-468 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus*.

10. A modified water-soluble glucose dehydrogenase having pyrrolo-quinoline quinone as a coenzyme wherein glutamate 277 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* or an amino acid residue corresponding to said residue is replaced by another amino acid residue.

11. A modified water-soluble glucose dehydrogenase having pyrrolo-quinoline quinone as a coenzyme wherein isoleucine 278 in the water-soluble PQQGDH derived from *Acinetobacter calcoaceticus* or an amino acid residue corresponding to said residue is replaced by another amino acid residue.

12. A modified water-soluble glucose dehydrogenase having pyrrolo-quinoline quinone as a coenzyme wherein at least one amino acid residue is replaced by another amino acid residue in the region defined by residues 268-289 or 448-468 in the amino acid sequence shown as SEQ ID NO: 1.

13. A PQQ glucose dehydrogenase comprising the sequence: Xaa8 Thr Ala Gly Xaa1 Val Gln Xaa2 Xaa3 Xaa4 Gly Ser Val Thr Xaa5 Thr Leu Glu Asn Pro Gly
wherein Xaa1, Xaa2, Xaa3, Xaa4, Xaa5 and Xaa8 represent any natural amino acid residue, provided that when Xaa1 represents Asn, Xaa2 represents Lys, Xaa3 represents Asp, Xaa4 represents Asp and Xaa5 represents Asn, then Xaa8 does not represent Asp.

14. A PQQ glucose dehydrogenase comprising the sequence: Ser Glu Gln Gly Pro Asn Ser Asp Asp Xaa6 Xaa7 Asn Leu Ile Val Lys Gly Gly Asn Tyr Gly Trp

wherein Xaa6 and Xaa7 represent any natural amino acid residue, provided that when Xaa6 represents Glu, Xaa7 does not represent Ile.

15. The modified glucose dehydrogenase of Claim 14 wherein glutamine 277 in the amino acid sequence shown as SEQ ID NO: 1 is replaced by another amino acid residue.

16. The modified glucose dehydrogenase of Claim 14 wherein isoleucine 278 in the amino acid sequence shown as SEQ ID NO: 1 is replaced by another amino acid residue.

17. A gene encoding the modified glucose dehydrogenase of any one of Claims 1-16.

18. A vector comprising the gene of Claim 17.

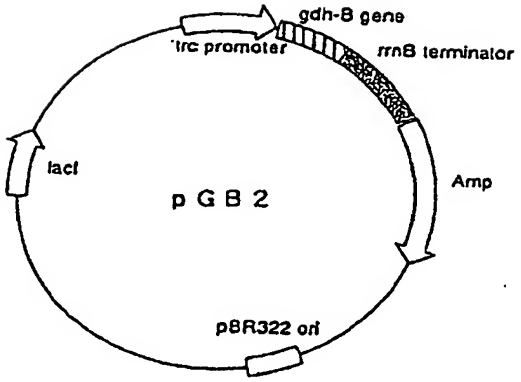
19. A transformant comprising the gene of Claim 17.

20. The transformant of Claim 19 wherein the gene of Claim 17 is integrated into the main chromosome.

21. A glucose assay kit comprising the modified glucose dehydrogenase of any one of Claims 1-16.

22. A glucose sensor comprising the modified glucose dehydrogenase of any one of Claims 1-16.

FIG. 1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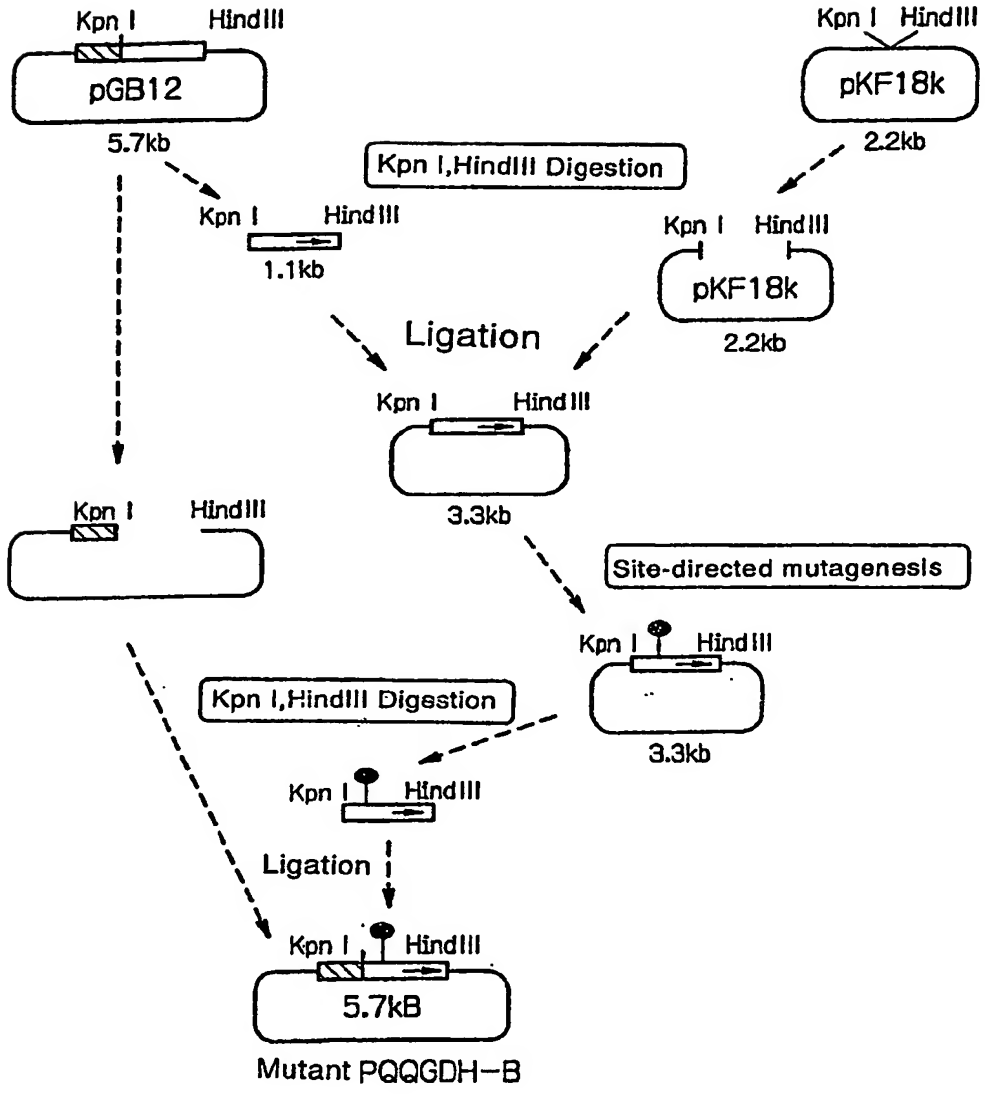
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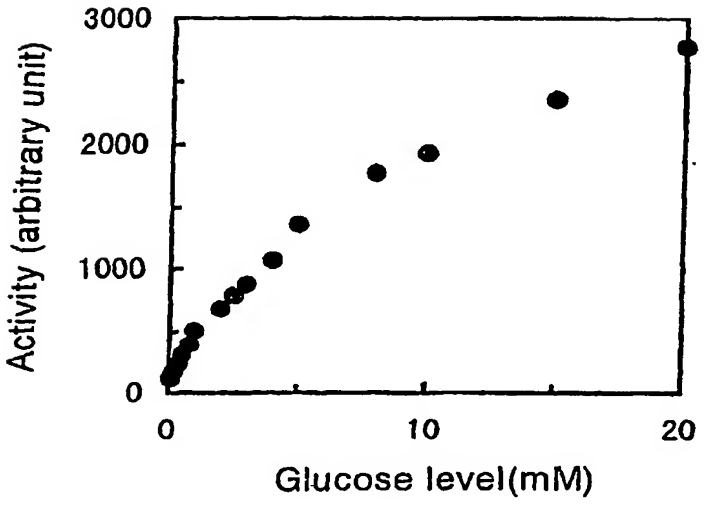
FIG. 3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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP00/02872

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl^7 C12N15/53, C12N15/63, C12N9/04, C12N1/19, C12Q1/32, C12M1/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl^7 C12N15/53, C12N15/63, C12N9/04, C12N1/19, C12Q1/32, C12M1/34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPI(DIALOG), BIOSIS(DIALOG), JICST FILE(JOIS)
GenBank/DDBJ/EMBL/Geneseq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	Igarashi S. et al. "Construction and Characterization of mutant Water-Souluble PQQ Glucose Dehydrogenases with altered Km Values Site-Directed Mutagenesis Studies on the Putative Active Site" Biochem. Biophys. Res. Commun. (November, 1999) Vol.264, No.3, pp.820-824	1-22
X	Yoshida, H. et al. "Engineering a chimeric pyrroloquinone glucose dehydrogenase: improvement of EDTA tolerance, thermal stability and substrate specificity" Protein Engineering (January, 1999) Vol.12, No.1 pp.63-70	1-2,17-22
X	JP, 10-243786, A (Koji Hayade), 14 September, 1998 (14.09.98) (Family: none)	1-2,17-22
A	Cleton-Jansen, A. M. et al. "Cloning, characterization and DNA sequencing of the gene encoding the Mr 50000 quinoprotein glucose dehydrogenase from Acinetobacter calcoaceticus" Mol. Gen. Genet. (1989) Vol. 217, NO. 2/3 pp.430-436	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 August, 2000 (01.08.00)	08 August, 2000 (08.08.00)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

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